Chapter 4

Examining the phosphorylation states of PtsN *in vivo* and further studies on the relationship between dephospho-PtsN and YcgO
Introduction

The relationship between the PtsP-PtsO-PtsN phosphorelay and \( \text{K}^+ \) metabolism was examined in chapter 3, with the observations that the growth inhibition displayed by the \( \Delta ptsN \) mutant in high [\( \text{K}^+ \)]\text{e} medium correlated with \( \text{K}^+ \) limitation mediated by YcgO. However as mentioned in chapter 3, PtsN in principle is capable of existing in two forms namely phospho and dephospho. Evidence albeit circumstantial was presented which indicated that it was the absence of dephospho-PtsN that was causal to the growth inhibition displayed by the \( \Delta ptsN \) mutant in high [\( \text{K}^+ \)]\text{e} medium. In order to delineate further the phosphorylation state of PtsN whose absence was responsible for the aforementioned phenotype, experimentation was undertaken that included (i) testing the effects of replacements of Histidine-73 of PtsN, the site of phosphorylation (Zimmer et al. 2008), with negatively charged amino acids, on its function, (ii) re-examining the role of SixA, a predicted phospho-histidine phosphatase on the PtsP-PtsO-PtsN pathway and (iii) determining the \textit{in vivo} phosphorylation states of PtsN. The results obtained in this chapter (i) yield further support to the notion that growth inhibition displayed by the \( \Delta ptsN \) mutant in medium of high [\( \text{K}^+ \)]\text{e} is correlated with the absence of dephospho-PtsN, (ii) indicate that \textit{in vivo} dephospho-PtsN may be limiting and (iii) provide evidence of a PtsO independent PtsP dependent pathway of phosphotransfer to PtsN.

Results

Effect of replacements of Histidine-73 on PtsN function

As described in chapter 3, the issue of the phosphorylation state of PtsN whose absence was causal to the \( \text{K}^+ \) sensitive phenotype (\( \text{K}^+ \text{S} \)) of the \( \Delta ptsN \) mutant, was addressed using plasmids expressing PtsN and PtsN bearing H73A, H73D and H73E substitutions. It was noted that
regardless of the substitutions all of the PtsN variants complemented the K^S of the ΔptsN mutant like the wild type PtsN, making interpretation of the obtained result not very straightforward (Sharma et al. 2016). It was thus necessary to examine effects of chromosomally encoded PtsN with the aforementioned replacements. Chromosomally expressed 3x FLAG epitope tagged versions of PtsN (PtsN^F) and its derivatives bearing H73A (PtsN^F_{H73A}), H73D (PtsN^F_{H73D}) and H73E (PtsN^F_{H73E}) amino acid substitutions were constructed in this study and their construction is schematically presented in the Fig. 4.1 and Fig. 4.2.

Towards this end, a DNA sequence encoding the 3x FLAG epitope was abutted to the codon encoding the carboxy-terminal amino acid, using approaches of recombineering (described in the materials and methods section). Following recombineering of the PCR product, Kan^R recombinants were obtained and one such recombinant designated GJ14906 was chosen and the ptsN locus in this recombinant was sequenced and designated ptsN^F. In GJ14906 the 3x FLAG epitope was correctly positioned, followed by the gene encoding the Kan^R determinant and expression of the downstream yhbJ gene was presumed to occur as a result of run-off transcription of the gene encoding the Kan^R determinant. An idealized RBS and an optimally placed ATG initiation codon from the ptsN distal end of the Kan^R cassette was fused to the second codon of yhbJ. The 3x FLAG tagged encoding ptsN from GJ14906 is identical to that constructed by Bahr et al. (2011) and was moved to other strains by P1 transduction employing a selection for the adjacent Kan^R marker and the encoded protein was designated PtsN^F.
Fig. 4.1: Schematic representation of the recombineering strategy used to append the DNA sequence encoding 3x FLAG epitope at 3’ end of ptsN. A PCR product bearing DNA sequences encoding the 3x FLAG epitope (red box), the Kan\textsuperscript{R} determinant (Kan), that is flanked by two FRT sites (blue arrows) is shown to recombine on chromosome via the H1 (Blue colored box) and H2 (violet colored box) homology regions at the 3’ end of ptsN. The direction of transcription of the gene encoding the Kan\textsuperscript{R} determinant is indicated by an interrupted red arrow. Faithful recombineering will generate a ptsN ORF ((ptsN\textsuperscript{F}) capable of encoding PtsN bearing a carboxy-terminal 3x FLAG epitope. The initiation (ATG) and the termination (TAG) codons of ptsN are indicated as are the initiation (ATG) and termination codons (TAA) of ptsN\textsuperscript{F}. The black box and the ATG codon (italicized) depict the RBS and the initiation codon located at the distal end of the Kan\textsuperscript{R} marker, provided for the expression of the downstream gene yhbJ. Recombination is depicted as occurring between double stranded DNA.

To engineer ORFs encoding PtsN\textsuperscript{F}_{H73A}, PtsN\textsuperscript{F}_{H73D} and PtsN\textsuperscript{F}_{H73E} on chromosome the procedure of recombineering was used. In the first step, a PCR product was used to replace DNA sequence between the 63\textsuperscript{rd} to the penultimate codon of ptsN with a DNA sequence encoding the Cm\textsuperscript{R} cassette (lacking the flanking FRT sites) by recombineering. The strain GJ17190 so generated was used as recipient strain for next step of recombineering. Using genomic DNA of the strain GJ14906 as template, PCR products were generated by overlap extension PCR that contained the nucleotide sequence encoding 3x FLAG tagged PtsN bearing
H73A, H73D and H73E substitution linked to the nucleotide sequence encoding the Kan\(^R\) marker, followed by the nucleotide sequence for downstream homology H2 from the second codon of \(yhbJ\). The sequence in the PCR product’s 5’ to the 63\(^{\text{rd}}\) codon served as the H1 homology sequence. Following recombineering all Kan\(^R\) recombinants recovered were Cm\(^S\), indicative of faithful recombineering. These Kan\(^R\) recombinants, which encoded PtsN\(^F\) bearing H73A, H73D and H73E substitutions were verified by sequencing and designated GJ17194, GJ17195 and GJ17196 respectively. The alleles encoding PtsN\(^F\)H73A (\(ptsN\(^F\)\)\(_{H73A}\)), PtsN\(^F\)H73D (\(ptsN\(^F\)\)\(_{H73D}\)) and PtsN\(^F\)H73E (\(ptsN\(^F\)\)\(_{H73E}\)) were introduced into other strains by P1 transduction selecting for the adjacent Kan\(^R\) marker.

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**Fig. 4.2:** Schematic representation of the strategy employed for generation of ORFs encoding 3x FLAG tagged PtsN bearing amino acid substitution H73A, H73D and H73E on chromosome. A PCR product bearing \(ptsN\) appended at its 3’ end with the nucleotide sequence encoding the 3x FLAG epitope (red box), along with the codon change (*)
responsible for generating the H73A, H73D and H73E substitutions linked to the Kan\(^R\) determinant (Kan), is shown to recombine on chromosome of an intermediate strain bearing the \(ptsN'::Cm\) allele, via the H1 (Blue colored box) and H2 (violet colored box) homology regions. The direction of transcription of the gene encoding the Kan\(^R\) determinant is indicated by an interrupted red arrow and the Kan\(^R\) determinant is flanked by two FRT sites (blue arrows). Faithful recombineering will generate a \(ptsN\) ORF (\(ptsN^F\)) capable of encoding PtsN bearing a carboxy-terminal 3x FLAG epitope and any one of the aforementioned substitutions. The recombineering will lead to replacement of the \(ptsN'::Cm\) allele. The initiation (ATG) and the termination (TAA) codons of \(ptsN^F\) are indicated. The black box and the ATG codon (italicized) depict the RBS and the initiation codon respectively, located at the distal end of the Kan\(^R\) marker, provided for the expression of the downstream gene \(yhbJ\). Recombination is depicted as occurring between double stranded DNA.

MC4100 and its derivative GJ18146 that expresses PtsN\(^F\) from its native chromosomal location grew equally on \(K_1\) and \(K_{115}\), media whereas its \(\Delta ptsN::\text{Kan}\) derivative JD17 displayed the \(K^S\) (Fig. 4.3). This indicated that abutting a 3x FLAG epitope to the carboxyl-terminus of PtsN did not impair its function. Derivatives of MC4100, GJ18147 and GJ18148 expressing \(PtsN^F_{H73A}\) and \(PtsN^F_{H73D}\) did not display the \(K^S\) at \(K_{115}\) but the MC4100 derivative GJ18149 expressing \(PtsN^F_{H73E}\) from chromosome displayed the \(K^S\) in \(K_{115}\) media (Fig. 4.3). Immunoblotting with Anti-FLAG antibodies demonstrated that levels of the PtsN variants in the aforementioned strains were comparable (Fig. 4.4).

![Immunoblotting](image)

Fig. 4.3: Growth phenotypes of strains bearing a chromosomal ORF, \(ptsN^F\), encoding PtsN bearing a carboxy-terminal 3x FLAG epitope and its derivatives bearing amino acid replacements of Histidine-73 with alanine (H73A), aspartate (H73D) and glutamate (H73E). Ten-fold serial dilutions of parent (MC4100), its \(\Delta ptsN::\text{Kan}\) mutant
(JD17) and its \( \text{ptsN}^F \) (GJ18146), \( \text{ptsN}_{H73A}^F \) (GJ18147), \( \text{ptsN}_{H73D}^F \) (GJ18148) and \( \text{ptsN}_{H73E}^F \) (GJ18149) containing derivatives, were spotted on K1 and K115 glucose agar plates.

Fig. 4.4: Levels of chromosomally encoded 3x FLAG tagged PtsN (\( \text{PtsN}^F \)) and its variants bearing amino acid substitutions H73A (\( \text{PtsN}_{H73A}^F \)), H73D (\( \text{PtsN}_{H73D}^F \)) and H73E (\( \text{PtsN}_{H73E}^F \)). \( A_{600} \) normalized whole cell lysates of exponentially grown cells of MC4100 (nil) and its PtsN, PtsN\( _{H73A}^F \), PtsN\( _{H73D}^F \), PtsN\( _{H73E}^F \) expressing derivatives GJ18146, GJ18147, GJ18148 and GJ18149 respectively, were separated on 12% SDS PAGE and transferred to PVDF membrane and probed with Anti–FLAG antibody. The image underneath the western blot is a portion of PVDF membrane stained with Amido black, as a gauge of equal loading.

**Delineating phosphorylation states of PtsN *in vivo***

Earlier studies have shown that direct determination of the phosphorylated states of \( \text{EIIA}^{Glc} \) the parologue of PtsN, *in vivo* is possible because the phospho and dephospho forms of \( \text{EIIA}^{Glc} \) display differential mobilities on SDS-PAGE gel (Nelson et al. 1986, Hogema et al. 1998). The dephospho form of \( \text{EIIA}^{Glc} \) migrates faster than its phosphorylated counterpart. In the case of PtsN, phosphorylation does not affect its mobility on SDS PAGE gel, however Lee et al. (2013) showed that the property of phosphorylation dependent mobility shift (PDMS) on SDS-PAGE gel can be imparted to PtsN with the introduction of the amino acid substitution K75D that lies near site of phosphorylation of PtsN namely His 73. Lee et al. (2013) have employed a plasmid
encoded K75D version of PtsN in their study. To detect phosphorylation states of PtsN, this study employed a chromosomally encoded 3x FLAG tagged version of PtsN bearing the K75D substitution that was constructed by recombineering and the procedure is schematically described in Fig. 4.5. A PCR product that extends from the nucleotide located 495 bases upstream of the start codon of ptsN and bearing the ptsN ORF with the codon change for the K75D substitution, followed by the nucleotide sequence encoding the 3x FLAG epitope and the KanR cassette and 45 nucleotides downstream homology of the next gene yhbJ (in that order) was generated by overlap extension PCR (Fig. 4.5)

Faithful recombineering of the codon change leading to the K75D substitution PtsN would introduce a new Sau3A1 restriction site. Following recombineering of the aforementioned PCR product, the obtained KanR recombinants were tested for the presence of the Sau3A1 polymorphism, by performing PCR of the ptsN ORFs followed by digestion of the PCR product with Sau3A1. Two types of KanR recombinants were obtained, one which retained the natural 75th codon (AAA) of ptsN and the other which bore the recombined nucleotide substitution (GAT) at 75th codon encoding PtsNFK75D (Fig. 4.5). The last base of the new Sau3A1 site namely C, would be contributed by first base of the 76th codon of ptsN that is CTG.
Fig. 4.5: Scheme for engineering a chromosomal ORF encoding carboxy-terminal 3x FLAG tagged derivative of PtsN bearing the K75D substitution. A PCR product bearing ptsN appended at its 3’ end with the nucleotide sequence encoding the 3x FLAG epitope (red box), along with the codon change (GAT) responsible for generating the K75D substitutions linked to the KanR determinant (Kan), is shown to recombine at the ptsN locus. Recombination between the H1 to H3 homology interval will generate the ORF ptsN^{F, K75D} encoding PtsN bearing the K75D substitution and tagged at its carboxy-terminus with the 3x FLAG epitope, whereas, that occurring between the H2 to H3 interval will generate the ptsN^{R} ORF retaining the natural 75th lysine codon. The direction of transcription of the gene encoding the KanR determinant is indicated by an interrupted red arrow and the KanR determinant is flanked by two FRT sites (blue arrows). The initiation (ATG) and the termination (TAA) codons of the two ptsN ORFs are indicated. The black box and the ATG codon (italicized) depict the RBS and the initiation codon respectively, located at the distal end of the KanR marker, provided for the expression of the downstream gene yhbJ. Recombination is depicted as occurring between double stranded DNA.
Growth of the strain GJ17027 encoding PtsN$_{K75D}^F$ on K$_1$ and K$_{115}$ was similar to its parent MC4100, whereas the ΔptsN::Kan mutant (JD17) displayed the K$_S^S$ indicating that PtsN$_{K75D}^F$ retained wild-type PtsN function (Fig. 4.6).

Fig. 4.6: Functionality of 3x FLAG tagged PtsN bearing the K75D substitution. Ten-fold serial dilutions of MC4100 (Parent) and its ΔptsN::Kan (JD17), ptsN$_{K75D}^F$ (GJ17027) bearing derivatives were spotted on K$_1$ and K$_{115}$ glucose agar plates.

PtsN phosphorelay is believed to involve PEP dependent sequential phosphotransfer of a phosphate moiety from PtsP to PtsN via PtsO (Powell et al. 1995, Reizer et al. 1996, Rabus et al. 1999). PtsN phosphorylation states were detected using PtsN$_{K75D}^F$ as probe, for strains cultivated in LB, K$_1$ and K$_{115}$ media. When the whole cell extract of GJ17027 (expressing PtsN$_{K75D}^F$), following growth in LB medium, was electrophoresed on SDS-PAGE followed by immunoblotting with Anti-FLAG antibodies, a slower moving form of PtsN was detected whereas only the faster moving form of PtsN was detected in the extract of its ΔptsP derivative, cultivated in the same medium (Fig. 4.7). The slower and the faster moving species of PtsN were inferred to be the phosphorylated and the dephosphorylated forms of PtsN respectively. These observations indicated that in LB medium phospho-PtsN is the abundant species, whereas dephospho-PtsN is the abundant species in the absence of PtsP in the same medium. Similar observations were recorded for strains grown in K$_1$ or K$_{115}$ media (Fig. 4.7). The observations
pertaining to phosphorylation states of PtsN in LB medium are consistent with those of Lee et al. (2013) where as those in $K_1$ and $K_{115}$ media are not and are discussed later. While it was expected that absence of PtsO would also lead to accumulation of dephospho-PtsN as the predominant species, it was noted that both the phosphorylation states of PtsN were detected in a strain lacking PtsO, regardless of the medium used for growth suggesting that there may be an alternate route of phosphorylation of PtsN in the absence of PtsO (Fig. 4.7).

Fig. 4.7: Phosphorylation dependent mobility shift of PtsN. Phosphorylation states of PtsN in vivo were detected following growth of the parent (GJ17027), its $\Delta ptsO::Cm$ (GJ18231) and $\Delta ptsP::Kan$ (GJ17066) derivatives in on LB, $K_1$ and $K_{115}$ media. All strains express a chromosomally encoded PtsN bearing the K75D substitution. $A_{600}$ normalized whole extracts of the indicated strains were separated on 15% SDS PAGE, transferred to PVDF membrane and probed with Anti–FLAG antibody. The asterisks and interrupted arrows indicate the relative mobilities of phospho-PtsN and of dephospho-PtsN respectively.

Re-examining the role of phosphohistidine phosphatase SixA on the PtsP-PtsO-PtsN phosphorelay

Recently Schulte and Goulian (2018) have proposed a role for the phosphohistidine phosphatase SixA in modulation of the PtsP-PtsO-PtsN phosphorelay. Specifically their studies have shown that (i) the absence of SixA leads to a $K^S$ that is suppressed by the absence of YcgO, (ii) the $K^S$ of the $\Delta sixA$ mutant correlates with YcgO mediated $K^+$ limitation, and (iii) overproduction of SixA leads to increased preponderance of dephospho-PtsN whose accumulation is PtsO
dependent. Schulte and Goulian (2018) have hypothesized that SixA directly dephosphorylates PtsO, leading to maintenance of a pool of dephospho-PtsN \textit{in vivo}. Overall there are many similarities between the phenotypes caused by the absence of PtsN and SixA and these were examined.

In the background of MC4100 that bears all the three major K\textsuperscript{+} uptake systems (Kdp, Trk and Kup), absence of SixA led to a weak K\textsuperscript{S} in comparison to that displayed by the K\textsuperscript{S} of \(\Delta\text{ptsN}\) mutant on K\textsubscript{115} medium. Deletion of \(\text{ycgO}\) suppressed the K\textsuperscript{S} of the \(\Delta\text{sixA}\) mutant and growth of the \(\Delta\text{ycgO}\) mutant JD466 was comparable to MC4100 (Fig. 4.8).

![Image](image)

\textbf{Fig. 4.8:} The K\textsuperscript{S} of \(\Delta\text{sixA}\) mutant and its suppression by deletion of \(\text{ycgO}\). Ten-fold serial dilutions of the parent (MC4100), its \(\Delta\text{ptsN}::\text{Kan}\) (JD17), \(\Delta\text{sixA}::\text{Kan}\) (GJ17037), \(\Delta\text{sixA} \Delta\text{ycgO}::\text{Kan}\) (GJ18237) and \(\Delta\text{ycgO}::\text{Kan}\) (JD466) derivatives were spotted on K\textsubscript{1}, K\textsubscript{20} and K\textsubscript{115} glucose agar plates.

In a strain background with Kdp as the sole K\textsuperscript{+} uptake system, the K\textsuperscript{S} caused by the absence of SixA was exacerbated, more so in a medium of intermediate \([\text{K}^+]_e\) (20 mM) than in a medium of high \([\text{K}^+]_e\) (115 mM). The K\textsuperscript{S} of the \(\Delta\text{sixA}\) mutant was also suppressed by the absence of YcgO and was weaker than that caused by the absence of PtsN (Fig. 4.9). The exacerbation of the K\textsuperscript{S} of the \(\Delta\text{sixA}\) mutant in the absence of constitutive K\textsuperscript{+} uptake systems was similar to that seen for \(\Delta\text{ptsN}\) mutant (described in chapter 3, Sharma et al. 2016) and offers
indirect support to the findings of Schulte and Goulian (2018) that the growth inhibition of the ΔsixA mutant in a synthetic medium of high [K\textsuperscript{+}]\textsubscript{e} is associated with K\textsuperscript{+} limitation.

![Image of bacterial growth on agar plates](image_url)

**Fig. 4.9:** Exacerbation of the K\textsuperscript{S} of the ΔsixA mutant in the absence of constitutive K\textsuperscript{+} uptake systems Trk and Kup and its suppression deletion of ycgO. Ten-fold serial dilutions of a strain with Kdp as sole K\textsuperscript{+} uptake system (JD624, parent), and its ΔaptsN::Kan (JD660), ΔsixA::Kan (GJ17038), ΔsixAΔycgO::Kan (GJ18238) and ΔycgO (JD696) derivatives were spotted on K\textsubscript{1}, K\textsubscript{20} and K\textsubscript{115} glucose agar plates.

One feature of the aforementioned studies was that in a strain background with Kdp as the only K\textsuperscript{+} transporter the K\textsuperscript{S} caused by the absence of SixA was more pronounced in a medium of intermediate (20 mM) [K\textsuperscript{+}]\textsubscript{e} than in a medium of high (115 mM) [K\textsuperscript{+}]\textsubscript{e}. Furthermore absence of YcgO alleviated the K\textsuperscript{S} in K\textsubscript{115} but not in K\textsubscript{20} media, The ΔaptsN::Kan derivative of a strain bearing only Kdp also displays a similar growth pattern (see chapter 3, Fig. 3.5, Sharma et al. 2016). This peculiar growth profile is probably related to the anomalous growth of a kdp\textsuperscript{+} trkA kup strain itself, that is known to experience partial K\textsuperscript{+} limitation in media of intermediate [K\textsuperscript{+}]\textsubscript{e}s (also discernible in Fig. 4.9), owing to the repression of the Kdp transporter by [K\textsuperscript{+}]\textsubscript{e} (Roe et al. 2000, Laermann et al. 2013). Growth of this strain in media of low (below 40 mM) [K\textsuperscript{+}]\textsubscript{e}s is dependent on the Kdp transporter whereas in media of [K\textsuperscript{+}]\textsubscript{e}s > 40 mM its growth is dependent on the TrkF K\textsuperscript{+} uptake activity (Roe et al. 2000, Laermann et al. 2013). Since dephospho-PtsN has been implicated in mediating optimal expression of the kdp operon (Lüttmann et al. 2009), and SixA is thought to maintain a pool of dephospho-PtsN in vivo (Schulte and Goulian 2018),
the greatly pronounced K$^S$ caused by the absence of SixA in K$_{20}$ medium is probably an exaggerated K$^+$ limitation caused due to sub-optimal kdp expression. It is assumed that in the kdp$^+$ trkA kup absence of SixA (or PtsN) leads to two distinct types of K$^+$ limitations, one occurring in media of intermediate (20 mM) [K$^+$]$_e$ and the other at high (115 mM) [K$^+$]$_e$. The former is thought to arise largely due to impaired kdp expression whereas the latter due to YcgO mediated K$^+$ efflux. The aforementioned rationale serves to explain the basis behind the absence of alleviation of the K$^S$ of the $\Delta$sixA mutation by the $\Delta$ycgO mutation in K$_{20}$ media.

To study the interplay of SixA with the PtsP-PtsO-PtsN phosphorelay, the effect of deletions of ptsO and ptsP on the K$^S$ of the sixA mutant was tested. When 0.2% glucose was used as the carbon source, the parent bearing Kdp as sole K$^+$ uptake system JD624, grew on K$_1$ and K$_{115}$ media but displayed reduced growth on K$_{20}$ because of [K$^+$]$_e$ mediated repression of the Kdp system (Roe et al. 2000, Laermann et al. 2013). The K$^S$ of $\Delta$sixA derivative was suppressed by deletions of both ptsP and ptsO on K$_{20}$ and K$_{115}$ media (Fig. 4.10). Since levels of dephospho-PtsN increase appreciably in ptsP and ptsO mutants (Bahr et al. 2011, Jahn et al. 2013, Lee at al. 2013, Fig. 4.7) and dephospho-PtsN is required for optimal kdp expression, (Lüttmann et al. 2009), the alleviation of the K$^S$ of the $\Delta$sixA mutant on K$_{20}$ medium, can be explained on the basis that in the absence of PtsP or PtsO, sufficient levels of dephospho-PtsN are available to mediate near-optimal expression of the kdp operon. The increased level of dephospho-PtsN is also thought to re-establish the proposed negative regulation of YcgO, thus explaining the suppression of the K$^S$ phenotype of the $\Delta$sixA mutant on K$_{115}$ medium, by the ptsP or ptsO deletions.
Fig. 4.10: The K^S of the ΔsixA mutant and its suppression by the absence of PtsP and PtsO on media containing 0.2% glucose as the carbon source. Ten-fold serial dilutions of parent (JD624), ΔsixA::Kan (GJ17038), ΔptsO::Kan (GJ18233), ΔptsP::Kan (GJ18234), ΔsixA ΔptsO::Kan (GJ18235) and ΔsixA ΔptsP::Kan (GJ18236) derivatives were spotted on K_1, K_20 and K_115 containing 0.2% glucose as carbon source.

When 0.2% glycerol was used as the carbon source, instead of glucose (as in Fig. 4.10), the parent JD624 (kdp^+ trkA kup) grew in K_1 and K_115 media but displayed very poor growth at K_20 which is attributed [K^+]_c mediated repression of Kdp system (Fig. 4.11, Roe et al. 2000, Laermann et al. 2013). This growth defect is more pronounced when glycerol is used as the carbon source in place of glucose. The K^S of the ΔsixA mutant on K_115 medium was also very pronounced. A likely explanation for this could be that since glycerol is a poorer carbon source, the mild inhibition of growth of JD624 in K_20 medium is further exacerbated due to lower growth rate, as is the K^S of the ΔsixA mutant in glycerol containing K_115 medium (Fig. 4.11). However an alternate possibility could be that since glycerol is a non-PTS carbon source, the phosphorylated versions of EI and Hpr that predominate in glycerol grown cultures, may contribute additionally to the phosphorylation of PtsN, via the phenomenon of cross-talk (Fig. 1.4) as has been shown by earlier studies (Powell et al. 1995, Bettenbrock et al. 2007, Zimmer et al. 2008, Lüttmann et al. 2015), thus increasing the steady state levels of phospho-PtsN. Since dephospho-PtsN is thought to be required for both optimal expression of kdp (Lüttmann et al. 2009) and for fettering the K^S caused by YcgO activity (Sharma et al. 2016), the exacerbation of
the growth defect of JD624 in K20 as well as that of the ΔsixA mutant in glycerol containing K115 medium may be attributed to limiting dephospho-PtsN.

Absence of PtsP alleviated the growth defect of JD624 in K20 medium, which is supportive of the notion that the poor growth of JD624 in the said medium is due to lowered levels of dephospho-PtsN. That, the ΔptsO mutant did not alleviate the poor growth of JD624 in K20 medium can be attributed to the presence of a significant pool of phospho-PtsN under this condition (also see Fig. 4.7).

The ΔsixA mutant displayed the Ks both on K20 and K115 and the one on K115 was alleviated by the absence of PtsO but not by the absence of PtsP (Fig. 4.11). In the context of growth in K115, medium, this observation is consistent with those of Schulte and Goulian (2018). They have used a strain bearing all the active K+ uptake systems and have proposed that cross talk between components of the carbohydrate PTS and the PtsP-PtsO-PtsN phosphorelay can serve to explain the differential effects of the ptsP and ptsO mutations in alleviating the Ks of the ΔsixA mutant. As described earlier, growth on non-PTS carbohydrates such as glycerol is associated with higher phosphorylation levels of PTS sugar proteins, in comparison to growth on a PTS sugar such as glucose (Bettenbrock et al. 2007). Given this, it is thought that in the ΔptsP mutant during growth on glycerol an additional source of phosphorylation, namely EI (PtsI), exists, that is lost (or attenuated) during growth on glucose (Schulte and Goulian 2018). In effect Schulte and Goulian (2018) implicate the EI-PtsO-PtsN phosphorelay as a contributor in generating phospho-PtsN that leads to Ks in the ΔptsP mutant. Furthermore, the alleviation of the Ks of the ΔsixA mutant by the absence of PtsO, on K115 medium implies that SixA exerts its effects by dephosphorylating PtsO, and confirms the observation of Schulte and Goulian (2018). The Ks of the ΔsixA mutant on K20 was not alleviated by the absence of PtsP, when glycerol
(Fig. 4.11) but not glucose (Fig. 4.10) was used as a carbon source. This observation may be explained on the basis that despite the absence of PtsP, dephospho-PtsN is limiting in the absence of SixA, owing to alternate routes of phosphorylation of PtsN. Since growth of the parent JD624 (kdp+ trkA kup) in K20 medium is dependent on Kdp function and dephospho-PtsN is required for optimal kdp expression (Lüttmann et al. 2009), the inability of the ΔsixA ΔptsP derivative of JD624 to grow on K20 medium can be attributed to limiting dephospho-PtsN. Lastly the ΔptsO mutation suppressed the Ks of the ΔsixA mutant in K115 medium but not on K20 medium, which can be accounted for if one assumes that the available level of dephospho-PtsN in the ΔsixA ΔptsO is enough to fetter YcgO but is not enough for optimal kdp expression.

![Image](image_url)

Fig. 4.11: Absence of suppression of the Ks of the ΔsixA mutant by a deletion of ptsP on media containing 0.2% glycerol as the carbon source. Ten-fold serial dilutions of parent (JD624), ΔsixA::Kan (GJ17038), ΔptsO::Kan (GJ18233), ΔptsP::Kan (GJ18234), ΔsixA ΔptsO::Kan (GJ18235) and ΔsixA ΔptsP::Kan (GJ18236) derivatives were spotted on K1, K20 and K115 containing 0.2% glycerol as carbon source.

**Discussion**

In this chapter the issue on the requirement of dephospho-PtsN as a negative regulator of YcgO activity was addressed. In that regard the phenotypes imparted by chromosomally encoded PtsN variants bearing replacements of histidine-73, the site of PtsN phosphorylation (Zimmer et al.
2008) with alanine, aspartate and glutamate, were tested. Of the three, only PtsN bearing the H73E substitution led to the K^S (Fig. 4.3). While the absence of K^S for the strain expressing PtsN_{H73A}, is in accord with the notion that dephospho-PtsN fetters YcgO activity, the opposing phenotypes displayed by strains expressing PtsN_{H73D} and PtsN_{H73E} at a first glance appear confounding since both were expected to behave as phosphomimetic versions of phospho-PtsN and yield the K^S. Another confounding issue pertains to an earlier observation of this study which noted that P_{trc} driven expression of PtsN bearing the H73A, H73D and in particular the H73E substitutions from multicopy plasmids, complemented the K^S of the ΔptsN mutant like the wild type PtsN (chapter 3, Sharma et al. 2016).

One way to rationalize this would be to assume that all of the aforementioned PtsN variants to differing extents retain features of a “bonafide dephosphoPtsN” molecule. Indeed, Lüttmann et al. (2009) have noted that expression of PtsN_{H73A} as well as PtsN_{H73D} from the P_{ara} promoter of the plasmid pBAD18 (Guzman et al.1995), led to enhanced expression of a kdp-lac transcriptional fusion in comparison to that of wild type PtsN. They have used this evidence (among others) to propose that dephospho-PtsN is required for optimal expression of the kdp operon and have implied that both PtsN_{H73A} and PtsN_{H73D} mimic dephospho-PtsN. In principle all the PtsN variants in question are incapable of being phosphorylated and therefore are potential protein mimics of dephospho-PtsN. If one assumes that PtsN bearing either the H73A or H73D substitution is closest to a bonafide dephospho-PtsN and the H73E substitution bearing derivative is the farthest, then overexpression of these proteins may lead to the predominance of the feature of dephospho-PtsN and hence would complement the ΔptsN mutant. If the aforementioned assumption is valid then absence or presence of the K^S by chromosomal expression of PtsN_{H73A}, PtsN_{H73D} and PtsN_{H73E} respectively may be explained (Fig. 4.3).
Dephospho-PtsN has been implicated in the optimal regulation of \( kdp \) expression as a factor that stimulates the activity of the KdpD kinase (Lüttmann et al. 2009). Furthermore, a recent study has shown that PtsN\(_{H73A}\) displayed a significantly higher affinity for the site of its interaction with KdpD, namely the cytoplasmic DHp domain of KdpD, in comparison to PtsN\(_{H73E}\). At least in the context of regulation of \( kdp \) expression the latter PtsN variant has been termed as phosphomimetic (Mörk-Mörkenstein et al. 2017). Nonetheless the aforementioned studies render the notion that dephospho-PtsN is a negative regulator of YcgO, less equivocal.

Perhaps more compelling evidence in support of the relationship between dephospho-PtsN and YcgO proposed in this work, concerns studies on the interplay between the phosphohistidine phosphatase SixA and the PtsP-O-N phosphorelay. Although originally reported by Schulte and Goulian (2018), it was necessary to test the effect of the \( \Delta \text{sixA} \) mutation, in order to establish its generality as a modulator of the PtsP-PtsO-PtsN phosphorelay. The essence of the study by Schulte and Goulian (2018) has been recapitulated in this chapter which is that the effect of SixA on the PtsP-PtsO-PtsN-YcgO axis is PtsO dependent and results obtained are consistent with the model proposed by Schulte and Goulian (2018) that SixA by dephosphorylating PtsO could maintain a pool dephospho-PtsN that fetters YcgO. Though not reported by Schulte and Goulian (2018), and going purely by phenotypes, the \( K_S \) of the \( \Delta \text{sixA} \) mutant was distinctly weaker than that of the \( \Delta \text{ptsN} \) mutant (Fig. 4.8 and Fig. 4.9). This suggests that there may be another PtsN specific phosphatase(s) whose activity in absence of SixA leads to a certain but reduced pool of dephospho-PtsN or that some amount of dephospho-PtsN may be generated in absence of SixA via spontaneous dephosphorylation of PtsN, under the conditions tested.
The aspect of obtaining direct visual estimates of the \textit{in vivo} phosphorylation states of PtsN was investigated by detection of differential mobility of phospho-PtsN and dephospho-PtsN, imparted by the K75D substitution in PtsN (Lee et al. 2013). These studies indicated that regardless of the medium employed phospho-PtsN is the major species in \textit{E. coli} that totally converts to dephospho-PtsN in the absence of PtsP. This indicates that dephospho-

PtsN may be limiting in the cell. Lee et al. (2013) have shown that the phosphorylation state of PtsN can be modulated by direct yet antagonistic actions of glutamine and $\alpha$-ketoglutarate, metabolites that signal nitrogen sufficiency or deficiency respectively, on the autophosphorylation of PtsP. Glutamine \textit{in vitro} was found to inhibit autophosphorylation of PtsP whereas $\alpha$-ketoglutarate stimulated this activity. Lee et al. (2013) further showed that this effect had a corresponding effect \textit{in vivo} on the phosphorylation states of PtsN in a manner dependent on nitrogen availability. Thus, in cells grown in a high nitrogen (20 mM) containing medium dephospho-PtsN was the major species and in those grown in a low (1mM) nitrogen containing medium, phospho-PtsN predominated. All media used in the current study are nitrogen rich media like LB and the nitrogen content in both K$_1$ and K$_{115}$ media is 8 mM, considered to be a nitrogen rich environment. The reason(s) therefore for this discrepancy is not clear though multiple reports indicate that by and large the major species of PtsN \textit{in vivo} in nitrogen rich media is phospho-PtsN (Bahr et al. 2011, Jahn et al. 2013, Lüttmann et al. 2015).

Both PtsP and PtsO are believed to act in the same pathway of phosphoryl transfer to PtsN (Powell et al. 1995, Rabus et al. 1999). Hence it was surprising to note that whereas PtsN was exclusively in the dephosphorylated state in the $\Delta$ptsP mutant, both the forms of PtsN could be detected in roughly equal proportion in the $\Delta$ptsO mutant (Fig. 4.7). Jahn et al. (2013) have also noted the above though they have not commented upon its possible physiological
significance. That the phenotype conferred by the ΔptsP mutation can be different (non-equal) from the ΔptsO mutation has been reported in the case of expression of the kdp operon (Lüttmann et al. 2009). Magnitudes of derepressed kdp-lac expression are different in the ΔptsP and ΔptsO mutants, with derepression being greater in the ΔptsP mutant (Lüttmann et al. 2009). As described earlier a strain bearing Kdp as the only K⁺ uptake system displays growth inhibition in a medium of intermediate [K⁺]ₑ (20 mM), particularly when glycerol was used as the carbon source (Fig. 4.11), that is known to correlate with K⁺ limitation, owing to repression of kdp expression and/or inhibition of Kdp activity by external K⁺ (Roe et al. 2000, Laermann et al. 2013). Relief from this growth defect was seen in a strain lacking PtsP but not PtsO (Fig. 4.11). This may be considered to be a reflection of the non-equal effects of the ΔptsP and ΔptsO mutations on kdp expression and is consistent with the observation of Lüttmann et al. (2009).

Therefore, it seems possible that there may exist another route of phospho-transfer to PtsN, one that is PtsP dependent but PtsO independent, involving another “PtsO like” protein. The most likely candidate that springs to mind is Hpr, the paralogue of PtsO in the sugar PTS. Multiple studies have implicated a cross talk between components of the sugar PTS and the PtsP-PtsO-PtsN phosphorelay (Powell et al. 1995, Rabus et al. 1995, Lüttmann et al. 2015, Schulte and Goulian 2018, Fig. 1.4). While cross talk in vitro has been demonstrated (Powell et al. 1995, Rabus et al. 1999), examples of those observed in vivo are apparent under conditions where the cells are cultivated in media containing non-PTS carbon sources such as LB (Zimmer et al. 2008) and glycerol (Schulte and Goulian 2018). Under these conditions components of sugar PTS are largely present in the phosphorylated state (reviewed in Postma et al. 1993, Deutcher et al. 2006). The former (Zimmer et al. 2008) is indicative of an EI-Hpr-PtsN phosphotransfer whereas the latter (Schulte and Goulian 2018) is indicative of an EI-PtsO-PtsN phosphotransfer. The
quality of the carbon source is also believed to influence phosphorylation but only in the absence of PtsP (Lüttmann et al. 2015). In the sense that Lüttmann et al. (2015) have shown that in the ΔptsP mutant, dephospho-PtsN predominates as long as cells are cultivated on PTS sugars whereas phospho-PtsN predominates following growth on non-PTS sugars, wherein both EI and Hpr are present largely in their phosphorylated forms. Lüttmann et al. (2015) have also noted that regardless of the carbon source as long as PtsP is present, phospho-PtsN is the major species in vivo, implying that PtsP is the major contributor to PtsN phosphorylation.

Two observations appear to exclude Hpr as the likely “PtsO like” candidate. Firstly, in vitro phosphorylation studies show that PtsP shows a strong specificity for PtsO and is incapable of phosphotransfer to Hpr (Rabus et al. 1999). Secondly, significant levels of phospho-PtsN were detected in the ΔptsO mutant in media containing glucose (Fig. 4.7), wherein dephospho-Hpr is the predominant species in vivo. Thus, it seems likely that a PtsO independent PtsP dependent route of phosphotransfer to PtsN exists involving another protein(s) that is not Hpr. Further studies are therefore required to examine this notion in greater detail.